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PRINCIPAL INVESTIGATOR: Lawrence A. Donehower, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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Lawrence A. Donehower, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail:
Larryd@bcm.tmc.edu

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13. ABSTRACT (Maximum 200 Words)

We have generated and characterized several mouse models to better understand the role of breast cancer associated genes in an experimentally manipulable context. In one model, we have examined Wnt-1 transgene-initiated mammary adenocarcinomas in the presence and absence of p53. In this model, we have identified seven differentially expressed genes which are dependent on p53 status. All of these genes are relevant either to cell cycle control or differentiation state and may be partially responsible for the p53-dependent difference in biological attributes. In a second set of studies, we have shown that Brca2 heterozygosity in a p53 null background accelerates mammary tumorigenesis compared to their Brca2 wild type counterparts, suggesting that mere reduction in Brca2 dosage is sufficient to accelerate tumorigenesis. Finally, we are studying the effects of a novel p53 target gene, Wip1, for its role in mammary tumorigenesis.

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Table of Contents

INTRODUCTION:

During the last year of this Academic Award Period, we have focused on several projects. The first project was the completion of our studies on the identification and characterization of p53-dependent mammary tumor markers. Using the Wnt-1/p53-deficient bitransgenic model, we examined gene expression patterns inWnt-1 initiated mammary tumors in the presence and absence of p53. We identified seven p53-dependent differentially expressed genes that fell either into the category of growth regulatory gene or differentiation marker gene (see accompanying reprint). Not surprisingly, p53-positive tumors showed increased expression of differentiation marker genes and negative growth regulatory genes, consistent with their more indolent, more differentiated biological characteristics. Our second project focused on the interactions of p53, Brca2, and Wnt-1 in two mammary tumor models. In our first experiments, we found that Brca2+/- mice did not exhibit accelerated tumorigenesis in the context of a Wnt-I transgene. However, in collaboration with Dr. Dan Medina, we showed that in the absence of p53, mammary glands with Brca2 heterozygosity incurred an increased number of tumors compared to Brca2 normal mammary glands in a mammary tumor susceptible Balb/c background. In a third set of experiments we are studying the functions of a new breast cancer-associated gene, Wip1, in normal animals and in mammary tumor models. We have generated Wip1 knockout mouse model and have shown defects in cell cycle control in cells derived from Wip1 null animals. We are crossing these mice to mammary tumor models to explore the role of Wip1 in mammary tumorigenesis and are exploring the role of Wip1 overexpression in mammary tumorigenesis.

RESEARCH ACCOMPLISHMENTS:

I. Experiments to detect p53-dependent alterations in gene expression in a mammary tumor model

During the past year, we completed our studies of the Wnt-1/p53 bitransgenic mammary tumor model (1-4). The p53 tumor suppressor is mutated or lost in 30-40% of human breast cancers and thus plays an important role in breast cancer formation and/or progression (5). To model the effects of p53 in a manipulable small animal model, we utilized the Wnt-1/p53-deficient mouse, which develops mammary cancers in a stochastic, but reasonably predictable fashion (1). We had previously showed that Wnt-1 transgenic p53+/+ mice developed tumors that arose with a longer latency, grew more slowly, showed more normal cell cycle control, more genomic stability, and more differentiatiated histopathology than their Wnt-1 p53-/- counterparts (1-3). Using several methods, including differential display and cDNA arrays, we identified seven genes that were consistently altered in expression in the p53-/- and p53+/+ mammary tumors. This work was published in a recent paper in *Oncogene* (see accompanying reprint) (4). All of these markers were confirmed by Northern blot analysis and by Western blot analysis where antibodies were available. All markers examined were confirmed to be differentially expressed at the protein level. Some of the identified differentially regulated genes were known differentiation markers for the mammary gland, including alpha smooth actin, cytokeratin 19, and kappa casein). Other differentially regulated genes were growth regulatory in function, such as p21WAF1, c-kit, and cyclin B1, and their expression correlated well with the differing growth properties of the p53-positive and p53-negative mammary tumors. Thus, while tumors can arise and progress in the presence of functioning wild type p53, p53 may directly or indirectly regulate expression of an array of genes that facilitate differentiation and inhibit proliferation, contributing to a more differentiated, slow growing, and genomically stable phenotype. Currently, we are extending these studies, using more complex cDNA chip arrays to uncover other p53-dependent alterations in gene expressions in the Wnt-1 mammary tumor model.

II. Effects of Brca2 heterozygosity on mammary tumorigenesis in two mammary tumor models

The BRCA2 tumor suppressor gene has been shown to be mutated in a significant fraction of inherited breast cancers in humans (6,7). While Brca2 null mice exhibit embryonic lethality, Brca2 heterozygous mice are normal yet do not exhibit any obvious tumor predisposition (8,9). In order to determine whether Brca2 heterozygosity can influence mammary tumor initiation or progression in a more permissible context, we crossed Brca2+/- mice to Wnt-1 transgenic mice and compared the mammary tumorigenesis rates in Wnt-1 transgenic Brca2+/+ and Brca2+/- female offspring. The tumor susceptibility curves for the Wnt-1/Brca2+/+ and the Wnt-1/Brca2+/- females were essentially superimposible and statistical analyses indicated that Brca2 heterozygosity did not confer increased mammary tumor susceptibility in the context of the Wnt-1 transgene.

In a second set of experiments in collaboration with Dr. Dan Medina at Baylor, we obtained Balb/c Brca2+/- mice from Dr. Roger Wiseman and Balb/c p53-/- mice from Dr. Dan Medina. The Balb/c inbred background is a mammary tumor susceptible background and Balb/c p53+/- mice show a much higher incidence of spontaneous mammary adenocarcinomas than the standard p53+/-

mice that are usually in a C57BL/6 background (10). We crossed Balb/c p53-/-
mice to Balb/c Brca2+/- mice and obtained p53-/- Brca2+/-, p53-/- Brca2+/+, and
p53+/-, Brca2+/- female offspring. We removed the mammary glands from 6-8
week old females of these three genotypes and transplanted them into the
mammary gland fat pads of normal Balb/c recipient females. We then monitored
these animals for spontaneous mammary tumors. In preliminary results shown
below in Figure 1, we found that while p53+/- Brca2+/- mammary glands did not
give rise to mammary tumors by 60 weeks after transplantation, p53-/- Brca2+/+
and p53-/- Brca2+/- did give rise to a large number of tumors. Moreover, the
incidence of p53-/- Brca2+/- mammary tumors was significantly higher than the
p53-/- Brca2+/+ tumors. We are following up these initial experiments by
examining the histopathology, remaining Brca2 allele status, and assays for
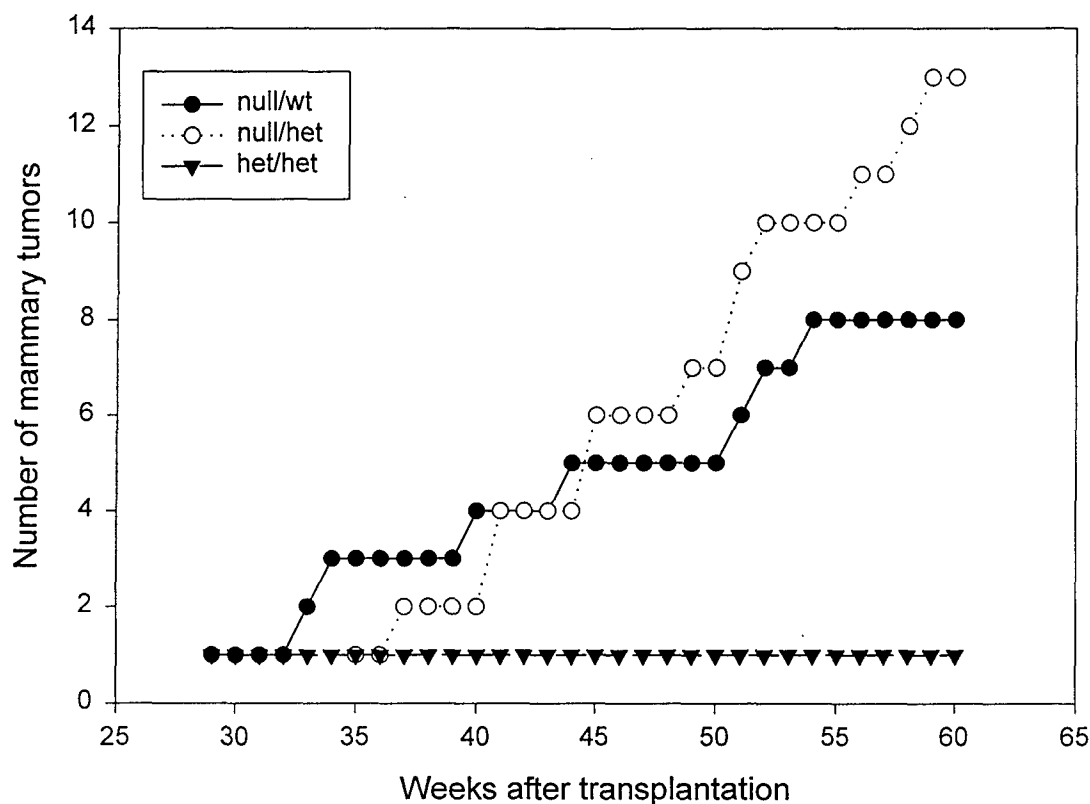tumor genomic instability.

Figure 1. Mammary tumor incidences in mammary glands derived from Balb/c
p53-/- Brca2+/-, p53-/- Brca2+/+, and p53+/-, Brca2+/- female mice.

III. Functional characterization of a novel breast cancer gene, Wip1

Several years ago our laboratory began collaborating with the laboratory of Ettore Appella at the National Cancer Institute on the characterization of a novel p53 target gene called the Wild type p53-Induced Phosphatase, or Wip1. Ionizing or ultraviolet radiation induces Wip1 in a p53-dependent manner (11). Recently, it has been shown that Wip1 dephosphorylates and inhibits p38 MAP kinase phosphorylation of Ser 33 and Ser 46 on p53 following cell stresses (12). Thus, Wip1 is induced by p53 and initiates a negative feedback loop response by causing a consequent inhibition of p53 activity. Recently, our collaborators have shown that Wip1 is amplified and overexpressed in human breast cancers (D. Bulavin and E. Appella, personal communication). Virtually all of these Wip1 overexpressing tumors retain wild type p53, suggesting a mechanism by which p53 activity is downregulated by increased Wip1, allowing cancer emergence.

To study Wip1 function, we have generated Wip1 knockout mice. We have shown that Wip1 is ubiquitously expressed in all tissues, but at very high levels in the round spermatid compartment of the testes (13,14). Mice lacking Wip1 are viable, but males show a reduced longevity and frequent runting (14). Wip1 null males also show reduced fertility and smaller reproductive organs. Interestingly, up to the age of two years, none of the Wip1 null animals exhibited tumors, consistent with a tumor resistance phenotype in the absence of Wip1 (14). According to the model cited above, Wip1 overexpression would be expected to inhibit p53 function, but the absence of Wip1 might augment p53 function and prevent tumors.

To corroborate this model, we have isolated fibroblasts from Wip1-/- and Wip1+/+ embryos and studied their behavior in culture. Interestingly, Wip1-/- mouse embryo fibroblasts (MEFs) exhibit reduced growth rates in culture, reduced plating efficiency, and show greatly delayed cell cycle progress, including reduced numbers of cells in S phase and mitosis (Figure 2A-C). Wip1-/- MEFs show early senescence, suggesting the possibility of an enhanced p53 response, consistent with the known ability of Wip1 to inhibit p53 activation (Figure 2D). We have corroborated the enhanced p53 response in preliminary experiments, showing that p53 protein levels are modestly increased, while phosphorylation of serine 15 is dramatically increased in Wip1-/- cells compared to Wip1+/+ cells. Phosphorylated serine 15 is a marker for activated p53 and thus it appears that the p53 response in Wip1-/- cells might be increased. This is consistent with the more robust G1 arrest response in IR-treated Wip1-/- MEFs compared to Wip1+/+ MEFs (Figure 2E).

To better understand the effects of Wip1 in breast cancer, we have initiated two experiments. First, we have begun crossing the Wip1-/- mice to Wnt-1 transgenic mice susceptible to mammary tumors. If the Wip1 gene is important in inhibiting p53 activity then the appearance of Wnt-1 initiated mammary tumors in the absence of Wip1 should be considerably delayed. Second, we will attempt to generate MMTV-Wip1 transgenic mice to examine the effects of Wip1 overexpression in the mammary gland. If these transgenic mice do not develop mammary tumors, we will then cross them to the Wnt-1 transgenic mice in an attempt to detect acceleration of Wnt-1 initiated mammary tumors in the presence of overexpressed Wip1. Examination of the biological and molecular properties of the mammary tumors from t hese models should provide further insights into the mechanisms by which Wip1 regulates p53 function and may influence the tumorigenesis process.

A

(Graph: Cell Number X 10^{-6} vs Days)

B

Wip1+/+

G1 -	56.9%
S -	20.6%
G2/M -	29.6%
M -	1.7%
G1/S ratio -	2.8
G2/M ration -	17.4

Wip1-/-

G1 -	48.7%
S -	16.8%
G2/M -	36.9%
M -	0.7%
G1/S ratio -	2.9
G2/M ration -	52.7

PI

C

(Bar graph: Colony Number for Wip1+/+ and Wip1-/-)

D

(Graph: Cell Number X 10^{-6} vs Passage Number)

E

(Bar graph: RATIO OF BRDU LABELING IRRADIATED/UNIRRADIATED for Wip1+/+, Wip1-/-, p53-/-)
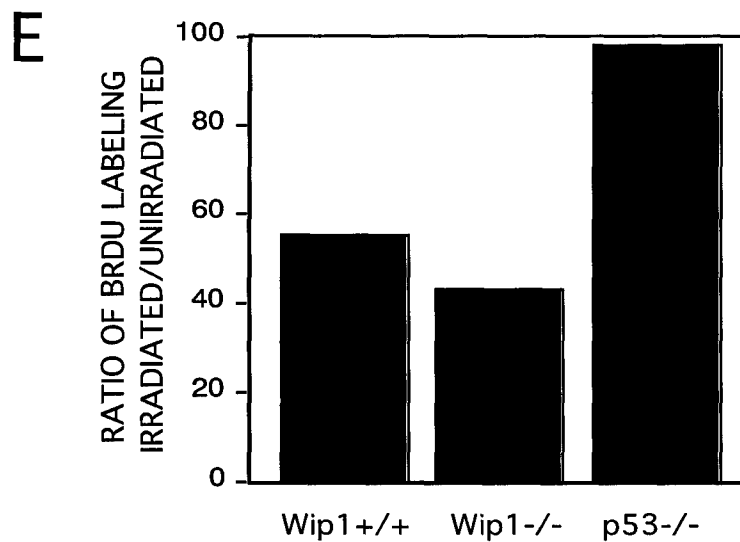
Figure 2. Growth phenotypes of *Wip1+/+* and *Wip1-/-* embryo fibroblasts. (A) Growth of 7.5 X 10^5 early passage MEFs derived from *Wip1-/-* and *Wip1+/+* embryos and monitored for growth over a period of 13 days. Representative growth curves are shown for *Wip1+/+* and *Wip1-/-* MEFs. The *Wip1-/-* MEFs consistently showed slower growth kinetics. (B) Flow cytometr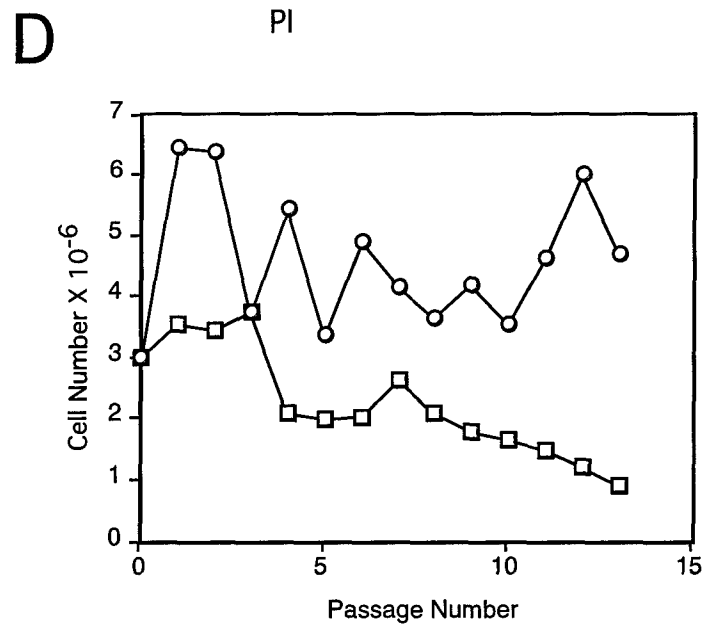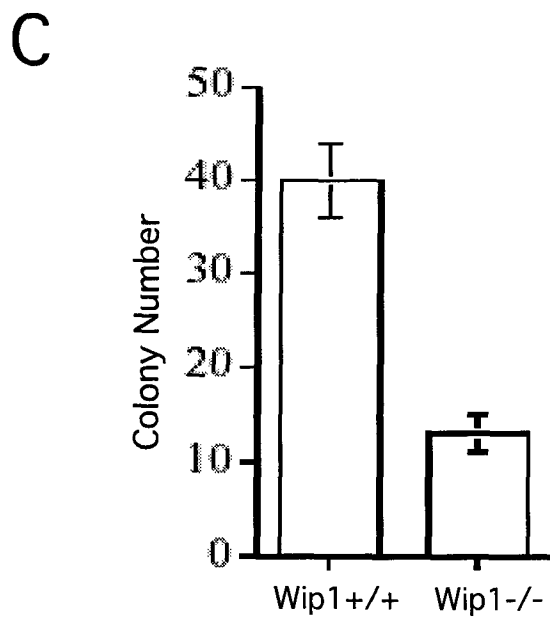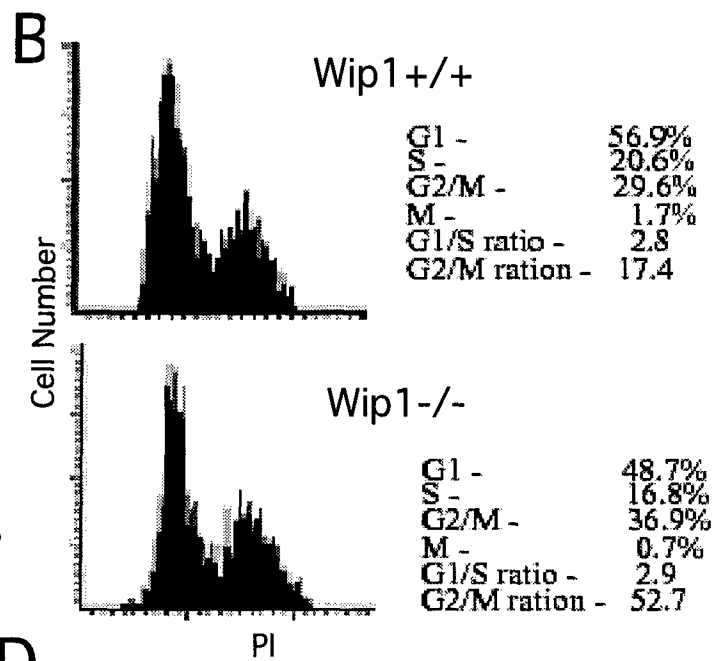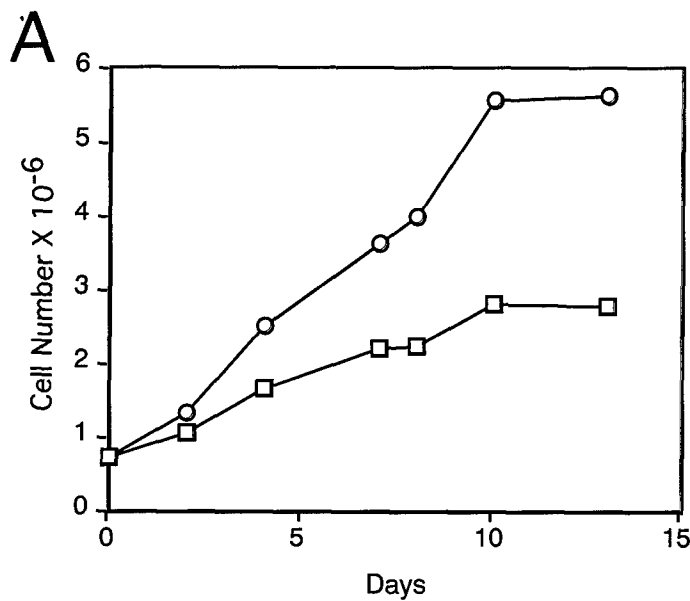y for DNA content and BrdU labeling on representative actively dividing early passage *Wip1+/+* MEFs (upper panel) and *Wip1-/-* MEFs (lower panel). Cells in M phase were determined by determination of the percentages of MEFs staining positive with fluorescent anti-histone H3 antibody. (C) Low density colony formation assay in which 10,000 *Wip1+/+* or *Wip1-/-* MEFs were plated on 100 mm tissue culture dishes, incubated two weeks and then fixed, stained and counted for colonies with more than 30 cells. (D) Long term passaging of *Wip1+/+* and *Wip1-/-* MEFs. Equal numbers of *Wip1+/+* and *Wip1-/-* cells were passaged every three days over 13 passages. Early during the passaging, *Wip1-/-* MEFs reached a terminal non-dividing state reminiscent of senescence, whereas wild type MEFs continued to divide rapidly through 13 passages. (E) G1 arrest response in *Wip1+/+*, *Wip1-/-*, and p53-/- MEFs. *Wip1+/+*, *Wip1-/-*, and p53-/- MEFs were synchronized in G1 and half of the cells of each genotype were treated with 5 Gy ionizing radiation. After BrdU addition, cells were harvested at 24 hours after irradiation and subjected to flow cytometry for DNA content and BrdU labelling. The ratio of BrdU labeled cells in treated vs. untreated MEFs for each genotype was determined. Note that p53-/- control MEFs are unaffected by irradiation, as expected.

APPENDIX:

List of Key Research Accomplishments:

(1)   Completion of experiments to detect p53-dependent alterations in gene expression in a mammary tumor model. Publication of results in Oncogene

(2)   Preliminary demonstration that Brca2 heterozygosity can augment mammary tumorigenesis in the absence of p53.

(3)   Generation of a Wip1 knockout mouse and the demonstration that Wip1 null cells have augmented p53 activity. Because Wip1 has recently been shown to be amplified and overexpressed in human breast cancers, further study of this gene may yield important insights into breast cancer etiology

List of Reportable Outcomes

(1)   One Published Paper (appended)

Cui, X. and Donehower, L.A. (2000). Differential gene expression in mouse mammary adenocarcinomas in the presence and absence of wild type p53. Oncogene 19: 5988-5996.

CONCLUSIONS:

I. **Experiments to detect p53-dependent alterations in gene expression in a mammary tumor model**

A. Mammary tumors can initiate and progress in the presence of wild type functional p53, but they arise later, grow slower, are more differentiated, exhibit more cell cycle control, and show more genomic stability.

B. p53 status in the Wnt-1-initiated mammary tumor model affects gene expression patterns. Seven differentially expressed genes were identified which were dependent on the presence or absence of p53.

C. The seven differentially expressed genes were either growth regulatory genes or differentiation markers, and their differential expression was consistent with the biological attributes of the tumors.

D. p53 may regulate tumor formation and progression through direct effects on regulation of proliferation and through less direct effects on differentiation-related genes.

II. **Effects of Brca2 heterozygosity on mammary tumorigenesis in two mammary tumor models**

A. In a Wnt-1-initiated mammary tumor model, Brca2 heterozygosity did not accelerate tumorigenesis.

B. In the absence of p53, Brca2 heterozygosity does increase mammary tumorigenesis rates, suggesting that in some contexts mere reduction of Brca2 dosage is sufficient to enhance tumorigenesis.

III. **Functional characterization of a novel breast cancer gene, Wip1**

A. Mice deficient for a novel p53 target gene, Wip1, that may be involved in human breast cancer, were generated, and show several unusual phenotypes.

B. Cells from Wip1 null mice display attenuated growth, consistent with current models that Wip1 inhibits p53 activity; thus, the absence of Wip1 may augment p53 activity, attenuating cell growth.

C. Wip1 null cells display increased levels of activated p53, again consistent with a p53 inhibitory role for Wip1.

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Differential gene expression in mouse mammary adenocarcinomas in the presence and absence of wild type p53

Xian-Shu Cui[1] and Lawrence A Donehower[*,1,2]

[1]*Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, TX 77030, USA;*
[2]*Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, TX 77030, USA*

The tumor suppressor p53 transcriptionally regulates a large number of target genes that may affect cell growth and cell death pathways. To better understand the role of p53 loss in tumorigenesis, we have developed a mouse mammary cancer model, the *Wnt-1* TG/p53 model. *Wnt-1* transgenic females that are p53 −/− develop mammary adenocarcinomas that arise sooner, grow faster, appear more anaplastic, and have higher levels of chromosomal instability than their *Wnt-1* transgenic p53 +/+ counterparts. In this study, we used several assays to determine whether the presence or absence of p53 affects gene expression patterns in the mammary adenocarcinomas. Most of the differentially expressed genes are increased in p53 +/+ tumors and many of these represent known target genes of p53 (p21[WAF1/CIP1], cyclin G1, alpha smooth muscle actin, and cytokeratin 19). Some of these genes (cytokeratin 19, alpha smooth muscle actin, and kappa casein) represent mammary gland differentiation markers which may contribute to the inhibited tumor progression and are consistent with the more differentiated histopathology observed in the p53 +/+ tumors. Several differentially expressed genes are growth regulatory in function (p21, c-*kit*, and cyclin B1) and their altered expression levels correlate well with the differing growth properties of the p53 +/+ and p53 −/− tumors. Thus, while tumors can arise and progress in the presence of functioning wild type p53, p53 may directly or indirectly regulate expression of an array of genes that facilitate differentiation and inhibit proliferation, contributing to a more differentiated, slow growing, and genomically stable phenotype. *Oncogene* (2000) 19, 5988–5996.

Keywords: p53; mouse mammary tumor; *Wnt-1*; p21[WAF1 CIP1]; c-*kit*; cyclin B1

Introduction

The p53 tumor suppressor gene is lost or mutated in over half of all human cancers (Levine, 1997; Lozano and Elledge, 2000). In addition, inactivation of p53 function without loss of p53 structural integrity may occur by a number of different mechanisms (Moll and Schramm 1998; Freedman *et al.*, 1999). Nevertheless, a significant fraction of human tumors arise and progress without incurring mutation or functional loss of p53 activity. In such tumors, retention of p53 activity has important clinical consequences. These tumors often have better prognoses and better responses to chemotherapeutic regimens (Kirsch and Kastan, 1998; Wallace-Brodeur and Lowe, 1999). Moreover, some tumor types with intact p53 exhibit less anaplastic histopathology, lower proliferation levels, and less chromosomal instability (Donehower, 1996).

The p53 protein is a transcriptional regulatory factor that responds to a number of cellular stresses, including DNA damage and activated cellular onco-genes (Giaccia and Kastan, 1998). The activated p53 protein can transactivate a number of genes involved either in cell cycle control or in cell apoptosis pathways (el-Deiry, 1998). One of the first identified targets of p53 was the p21[WAF1 CIP1] cyclin-dependent kinase inhibitor, which directly interacts with G1 cyclin-cdk complexes and inhibits their activity, and thus is an important component of the p53-mediated G1 arrest checkpoint (el-Deiry *et al.*, 1993; Harper *et al.*, 1993). Another important p53 target relevant to the apoptotic function of p53 is bax, a pro-apoptotic protein (Miyashita and Reed, 1995). Recently, the introduction of large scale screening technologies has greatly increased the number of known p53 targets. Using techniques such as serial analysis of gene expression (SAGE) and cDNA array analyses, a library of genes have been assembled that are either upregulated or downregulated by p53 (Polyak *et al.*, 1997; Yu *et al.*, 1999; Zhao *et al.*, 2000). Some of these genes regulate cell growth or death control, but others appear to be involved in physiological processes not directly related to growth or death (Polyak *et al.*, 1997; Zhao *et al.*, 2000). Moreover, there is a great deal of heterogeneity in the response of p53 target genes. A number of factors influence the types of p53-responsive genes that are activated or repressed, including p53 levels, the nature of the cellular stress, and the cell type being studied (Yu *et al.*, 1999; Zhao *et al.*, 2000).

While the SAGE and cDNA array screens have provided powerful tools for the identification of novel p53 target genes, there are potential limitations in the use of such screens. Generally, very high levels of p53 are often produced, which may fail to identify target genes regulated by physiological levels of p53. In addition, cancer cell lines of various types are often used and these cells have other genetic defects which may prevent identification of bona fide p53 targets. Finally, the experiments are performed in cell culture, and so targets may be missed that result from the activation of p53 in its normal *in-vivo* context.

To circumvent some of these potential limitations and identify genes regulated by p53 *in vivo* which might be directly relevant to tumorigenesis, we have utilized a murine mammary cancer model, the *Wnt-1* TG/p53 mouse. *Wnt-1* TG mice contain several copies of a germline *Wnt-1* oncogene driven by a mammary gland specific mouse mammary tumor virus promoter (Tsukamoto *et al.*, 1988). The female *Wnt-1* transgenic mice develop early mammary gland hyperplasia and usually succumb to mammary adenocarcinomas between the ages of 3 and 12 months. In order to determine the effects of p53 dosage on mammary tumorigenesis in this model, we crossed the *Wnt-1* TG mice to p53-deficient mice and the *Wnt-1* transgenic female offspring were monitored for mammary tumors in the presence and absence of p53 (Donehower *et al.*, 1995). As shown in Table 1, the absence of p53 (p53−/−) in the presence of the *Wnt-1* transgene resulted in mammary tumors that appeared sooner, grew faster, displayed less differentiated and more anaplastic histopathology, and exhibited more chromosomal instability than their *Wnt-1* TG p53+/+ counterparts (Donehower *et al.*, 1995; Jones *et al.*, 1997). Moreover, these differences in tumorigenic phenotypes were likely to be due directly to p53 status, since the p53 gene was not mutated or suppressed in the p53+/+ tumors (Donehower *et al.*, 1995).

Because the p53+/+ and p53−/− *Wnt-1* TG mice generate the same type of mammary adenocarcinomas, we decided to compare their gene expression patterns on the hypothesis that differences in gene expression might be relevant to p53 status and the observed tumorigenic phenotypes. While we believe there are a number of advantages of our *in-vivo* tumorigenesis model (e.g. a closer approximation to real physiological conditions), there may also be at least three potential limitations not encountered in the *in-vitro* screens. First, mammary tumors are heterogeneous and not composed solely of tumor cells. They are a mixture of epithelial tumor cells, myoepithelial and stromal components, adipose cells, and blood vessels. However, we have found that the epithelial tumor component usually predominates and thus the other nontumor components should not obscure any strong differences in gene expression. Second, despite being of identical histopathological type, intertumoral variation may be significant and apparent differences might be due to such variation rather than p53 status. To address this problem we analysed expression patterns in five to eight different tumors of each p53 genotype. Thus, if all or almost all p53+/+ tumors show higher expression levels of a particular gene than is seen in their p53−/− counterparts, then this difference is likely to be significant. Finally, comparison of end stage p53+/+ and p53−/− tumors will not necessarily identify direct p53 targets. Instead, secondary p53 targets or genes altered in expression due to other genetic changes might be detected. The discovery that many of the differentially regulated genes in our tumor model are known p53 target genes has reassured us that, in many cases, the differential expression is likely to be due directly to p53 expression levels.

Using several different approaches, we show here that, in addition to altering biological and genetic properties of mammary adenocarcinomas, p53 status affects gene expression patterns. At least seven differentially expressed genes have been identified in comparing p53+/+ and p53−/− tumors (Table 2). Some of the differentially expressed genes are clearly related to growth control, while others appear to be differentiation markers. The observed differential expression patterns of particular genes fit well with the biological properties of the parental tumors, suggesting that these genes may be a cause rather than an effect of the tumor phenotype. Thus, these results may provide further insights into the role of p53 target genes in the pleiotropic biological effects associated with tumorigenesis.

Table 1 Biological and genetic properties of *Wnt-1* TG p53+/+ and *Wnt-1* TG p53-/- mammary adenocarcinomas

Property	p53+/+	p53−/−
Tumor type	Mammary adenocarcinoma	Mammary adenocarcinoma
50% tumor incidence	22.5 weeks	11.5 weeks
100% tumor incidence	40 weeks	15 weeks
Mean tumor growth rate	800 cu. mm/wk	3400 cu. mm/wk
Mean percentage of mitotic tumor cells	0.0027	0.0070
Mean percentage of apoptotic tumor cells	0.32	0.48
Percentage of tumors with abnormal chromosomes	33	100
Mean number of abnormal chromosomes per tumor	0.3	1.7
Histopathological appearance	Uniform nuclei, differentiated, more stroma	Anaplastic, undifferentiated, less stroma

Table 2 Differentially expressed genes in *Wnt-1* TG p53+/+ and *Wnt-1* TG p53−/− mammary adenocarcinomas

Gene	ID method	p53+/+ levels	Mean difference	P-value	Protein	p53 target	Function/Marker
Alpha smooth muscle actin	Diff. Display	Increased	4.25	<0.001	Yes	Yes	Myoepithelial marker
Kappa casein	Diff. Display	Increased	3.18	0.045	ND	No	Luminal cell epithelial marker
c-*kit*	cDNA Array	Increased	2.38	0.01	Yes	No	Receptor tyrosine kinase
Cytokeratin 19	cDNA Array	Increased	2.73	0.003	Yes	Yes	Epithelial cell intermediate filament marker
P21$^{WAF1/CIP1}$	Northern	Increased	2.27	0.01	ND	Yes	Cdk inhibitor, growth marker
Cyclin B1	RNAse Prot	Decreased	2.31	0.04	Yes	Yes	Mitotic cyclin, growth marker
Cyclin G1	RNAse Prot	Increased	1.79	0.04	Yes	Yes	Function unclear poss. growth marker

Results

Differentially expressed genes identified by differential display PCR

To identify differentially expressed genes in Wnt-1 TG p53+/+ and Wnt-1 TG p53−/− tumors, we utilized several different types of screening methods. RNA differential display and cDNA array methods were used to randomly screen the tumors for differentially expressed genes. In addition, Northern blot and RNAse protection assays were employed to investigate specific known candidate genes. Each of these approaches revealed differentially expressed genes. Our first set of experiments employed RNA differential display PCR to screen tumor RNAs from Wnt-1 TG p53+/+, Wnt-1 TG p53+/− LOH, and Wnt-1 TG p53−/− tumors (Liang and Pardee, 1992). The Wnt-1 TG p53+/− LOH tumors are null for p53 because the remaining p53 wild type allele has been deleted during mammary tumorigenesis (Donehower et al., 1995). A number of candidate fragments were identified by this PCR-based method. An example of a fragment specific for the p53+/+ tumor RNAs is shown in Figure 1a. All fragments identified in this assay were at increased levels in the p53+/+ tumors. These p53+/+ specific fragments were then excised from the gel, reamplified with the appropriate differential display primers, labeled with ^{32}P and used as probes on Northern blots containing total RNAs from five p53+/+ and five p53−/− tumors. Two separate differential display

fragments consistently showed higher hybridization levels in the five p53+/+ tumors. These fragments were cloned and sequenced. The sequences were then compared with the GenBank database and were shown to be identical to two murine genes: alpha smooth muscle actin and kappa casein. The murine cDNA sequences of these two genes were then obtained and used to probe a Northern blot containing total RNAs from eight p53+/+ mammary adenocarcinomas and eight p53−/− adenocarcinomas (Figure 1b). Note that while there is heterogeneity in the RNA levels of each gene from tumor to tumor, when the hybridization levels are quantitated and normalized to control probe (GAPDH) hybridization intensity, significant increases in RNA levels of these genes are observed in p53+/+ tumors. Alpha smooth muscle actin RNA levels were on average 4.2-fold higher in p53+/+ tumors compared to p53−/− tumors. Kappa casein had a mean increase of 3.2-fold compared to p53−/− tumors. These differences were significant at the 0.05 level as measured by t-test.

Differentially expressed genes identified by cDNA array analysis

As an adjunct to the differential display analyses, we probed array filters containing 588 murine cDNAs (from Clontech) with ^{32}P-labeled cDNA probes prepared from mRNA derived from either p53+/+ or p53−/− tumors. There were two genes that consistently showed differential expression by this method: c-kit and cytokeratin 19 (Figure 2a). Both genes showed higher levels of hybridization in the p53+/+ tumors. cDNAs from cytokeratin 19 and c-kit were labeled with ^{32}P and hybridized to Northern blots containing mRNAs from multiple p53+/+ and p53−/− tumors. Again, while there was considerable heterogeneity in expression levels, the c-kit and cytokeratin 19 genes averaged 2.4- and 2.7-fold increases in expression in p53+/+ tumors compared to p53−/− tumors (Figure 2b and data not shown). These differences were found to be significant by t-test.

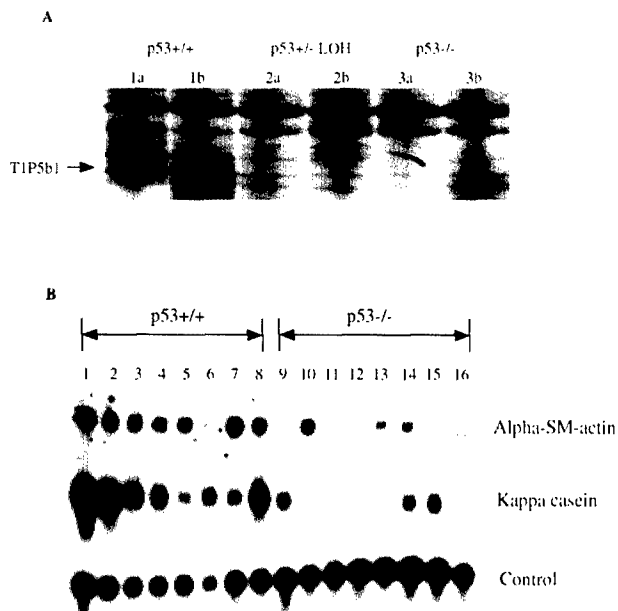**Figure 1** Differentially expressed genes in Wnt-1 TG p53+/+ mice and Wnt-1 TG p53−/− mice identified by differential display PCR and confirmed by Northern blot hybridization. (a) A representative differential display PCR product (TIP5b1, arrow) expressed at higher levels in the Wnt-1 TG p53+/+ tumors compared to either Wnt-1 TG p53+/− LOH or Wnt-1 TG p53−/− tumors. (b) Northern blot results of two genes (alpha smooth muscle actin and kappa casein). Eight different tumor RNA samples from each Wnt-1 TG genotype (p53+/+ and p53−/−) were loaded. After normalizing to the control RNA (GAPDH) signal, significant increases in RNA levels of these two genes are observed in Wnt-1 TG p53+/+ tumors

Examination of known p53 target genes

Those p53 target genes known to regulate growth control were obvious candidates for analysis. The prototype p53 target gene is p21$^{WAF1 CIP1}$, a cyclin-dependent kinase inhibitor (el-Deiry et al., 1993; Harper et al., 1993). Northern blot analysis of p53+/+ and p53−/− tumor RNAs using a murine p21$^{WAF1 CIP1}$ cDNA probe revealed that the p53+/+ tumors averaged 2.3-fold higher levels of p21 compared to the p53−/− tumors (Figure 2c), consistent with the reduced growth rates observed in the p53+/+ tumors. These differences were shown to be statistically significant.

Other important cell cycle regulatory proteins are the cyclins. At least two of these, cyclin B1 and cyclin G1, have been shown to be regulated by p53 (Innocente et al., 1999; Taylor et al., 1999; Okamoto and Beach, 1994). Cyclin B1 appears to be directly repressed by p53 and cyclin G1 has been shown to be upregulated by wild type p53. The cyclin mRNA levels were assessed in p53+/+ and p53−/− tumors by RNAse protection assay using kits specific for murine cyclin
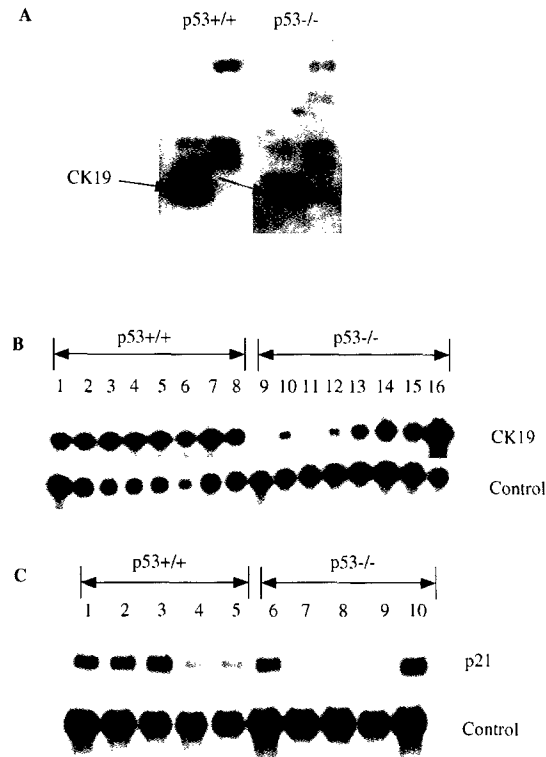
Figure 2 Differentially expressed genes in *Wnt-1* TG p53+/+ and *Wnt-1* TG p53−/− mammary tumors identified by cDNA array analysis and candidate gene Northern blot analyses. (a) Representative array showing a strong signal for cytokeratin 19 (CK19) in the *Wnt-1* TG p53+/+ sample compared to the *Wnt-1* TG p53−/− sample. (b) Northern blot hybridization of cytokeratin 19 (CK19) with eight tumor samples from each *Wnt-1* TG p53 genotype. After normalization of hybridization signals to control RNA (GAPDH) signals, CK19 expression is significantly higher on average in *Wnt-1* TG p53+/+ tumors. (c) Differential expression of the p53-target gene, p21$^{WAF1/CIP1}$, in *Wnt-1* TG p53+/+ and *Wnt-1* TG p53−/− mammary tumors. Northern blot hybridization shows five tumor RNA samples from each p53 genotype loaded for comparison. After normalizing to control (GAPDH) mRNA levels, p21$^{WAF1/CIP1}$ shows significantly higher expression in *Wnt-1* TG p53+/+ tumors than in *Wnt-1* TG p53−/− tumors

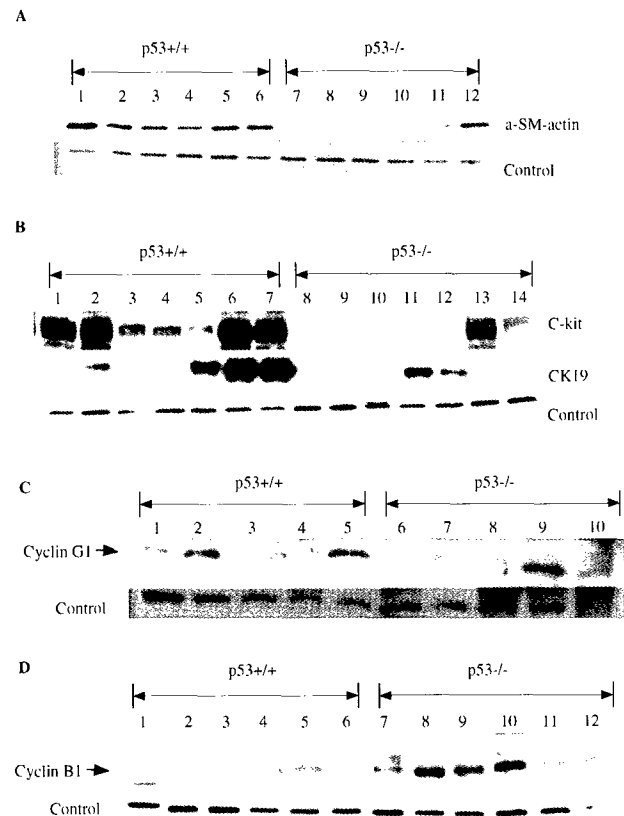**Figure 3** Differential expression of proteins in *Wnt-1* TG p53+/+ and *Wnt-1* TG p53−/− mammary tumors as assayed by Western blot analyses. Tumor lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to nylon membranes and immunoblotting with antibodies to the various differentially expressed proteins. Each blot was then stripped and immunoblotted with an antibody to vertebrate actin, which served as a loading control. (a) Alpha smooth muscle actin protein expression in six *Wnt-1* TG p53+/+ and six *Wnt-1* TG p53−/− mammary tumor lysates. Alpha smooth muscle actin levels were elevated in *Wnt-1* TG p53+/+ tumors compared to *Wnt-1* TG p53−/− tumors. (b) c-kit and cytokeratin 19 (CK19) proteins in seven *Wnt-1* TG p53+/+ and seven *Wnt-1* TG p53−/− mammary tumor lysates. The *Wnt-1* TG p53+/+ tumors had higher levels of c-kit and CK19 proteins on average compared to their *Wnt-1* TG p53−/− counterparts. (c) Cyclin G1 levels in five *Wnt-1* TG p53+/+ and five *Wnt-1* TG p53−/− mammary tumor lysates. *Wnt-1* TG p53+/+ tumors exhibited higher mean levels of cyclin G1 than *Wnt-1* TG p53−/− tumors. (d) Cyclin B1 protein levels in six *Wnt-1* TG p53+/+ and six *Wnt-1* TG p53−/− mammary tumor lysates. *Wnt-1* TG p53−/− tumors exhibited higher mean levels of cyclin B1 than *Wnt-1* TG p53+/+ tumors

mRNAs. Interestingly, the only cyclins to show significant differential expression after normalization to the GAPDH control RNA were cyclin B1 and cyclin G1 (data not shown). The p53−/− tumors averaged 2.3-fold higher cyclin B1 than p53+/+ tumors and p53+/+ tumors showed 1.8-fold elevated levels of cyclin G1 compared to p53−/− tumors. Again, these differences were found to be significant by *t*-test.

Differential protein expression levels

To correlate protein expression levels with the differentially expressed RNAs in the tumors, we performed Western blot analyses on extracts from p53+/+ and p53−/− tumors. Figure 3a shows that all of the p53+/+ tumors show high levels of alpha smooth muscle actin while only one of the six p53−/− tumors shows comparably high protein levels. Likewise, c-kit and cytokeratin 19 protein levels were generally higher in the p53+/+ tumors than their p53−/− counterparts, consistent with the earlier RNA results (Figure 3b). p53+/+ tumors also showed higher levels of cyclin G1 than p53−/− tumors

(Figure 3c). Finally, cyclin B1 protein levels were higher in the majority of p53−/− tumors than in p53+/+ tumors (Figure 3d), again correlating well with the RNA data for this gene.

Immunohistochemistry on tumor sections

The high expression of alpha smooth muscle actin in the p53+/+ tumors was of interest because this protein is known to be an important marker for the myoepithelial compartment of the mammary gland (Gugliotta *et al.*, 1988). To confirm whether the higher alpha smooth muscle actin levels indicated a higher number of myoepithelial cells in the p53+/+ tumors, we performed immunohistochemistry with alpha

smooth muscle actin antibodies on fixed sections of p53+/+ and p53−/− tumors. As shown in Figure 4, the staining patterns in the p53+/+ tumors reveal staining around the more organized glandular structures, consistent with a myoepithelial cell localization. In contrast, the staining for alpha smooth muscle actin in the p53−/− tumors is not only reduced in intensity and frequency, but appears in a more random disorganized pattern associated with the tumor stroma. These results are consistent with a more differentiated structural organization in the p53+/+ tumors than in their p53−/− counterparts.

Discussion

We believe that the *Wnt-1* TG/p53 model provides a number of advantages for mechanistic studies on the role of p53 in tumorigenesis. Virtually all of the *Wnt-1* TG females develop only mammary adenocarcinomas within 2–9 months either in the presence or absence of p53. In this model, the absence of p53 has been shown to dramatically alter the biological and genetic properties of the *Wnt-1*-initiated adenocarcinomas. An important question is whether the more aggressive and malignant characteristics of the p53−/− tumors are a direct result of the absence of p53 or an indirect result of the genomic instability promoted by the lack

of p53. In this latter scenario, the driving force for tumor initiation and progression would be the increased rate of cooperating genetic lesions in the p53−/− tumors. However, if p53 were playing a more active role in inhibition of tumor growth through its transcriptional regulatory function, then upregulation of p53 growth inhibitory targets and downregulation of p53 growth stimulatory targets might be observed. In fact, the increase in p21 and cyclin G1 levels and decreased cyclin B1 levels observed in the p53+/+ tumors are consistent with a direct role for p53 in modulating tumor growth rates. Moreover, the upregulation of several differentiation markers in the p53+/+ tumors, some direct targets of p53, suggests that these genes may be contributing to some of the biological properties of the tumors. Finally, the increased activities of the known p53 target genes in the p53+/+ tumors indicates that p53 signaling pathways are intact in these tumors, and that tumors can readily arise in this model in the presence of functional p53.

The differentially expressed genes that have been identified in this model fall generally into two categories, growth regulatory genes (p21, cyclin G1, cyclin B1, and c-*kit*) and differentiation markers (alpha smooth muscle actin, kappa casein, and cytokeratin 19). Some of the other categories of p53 target genes found in cell based screens, such as apoptosis-related
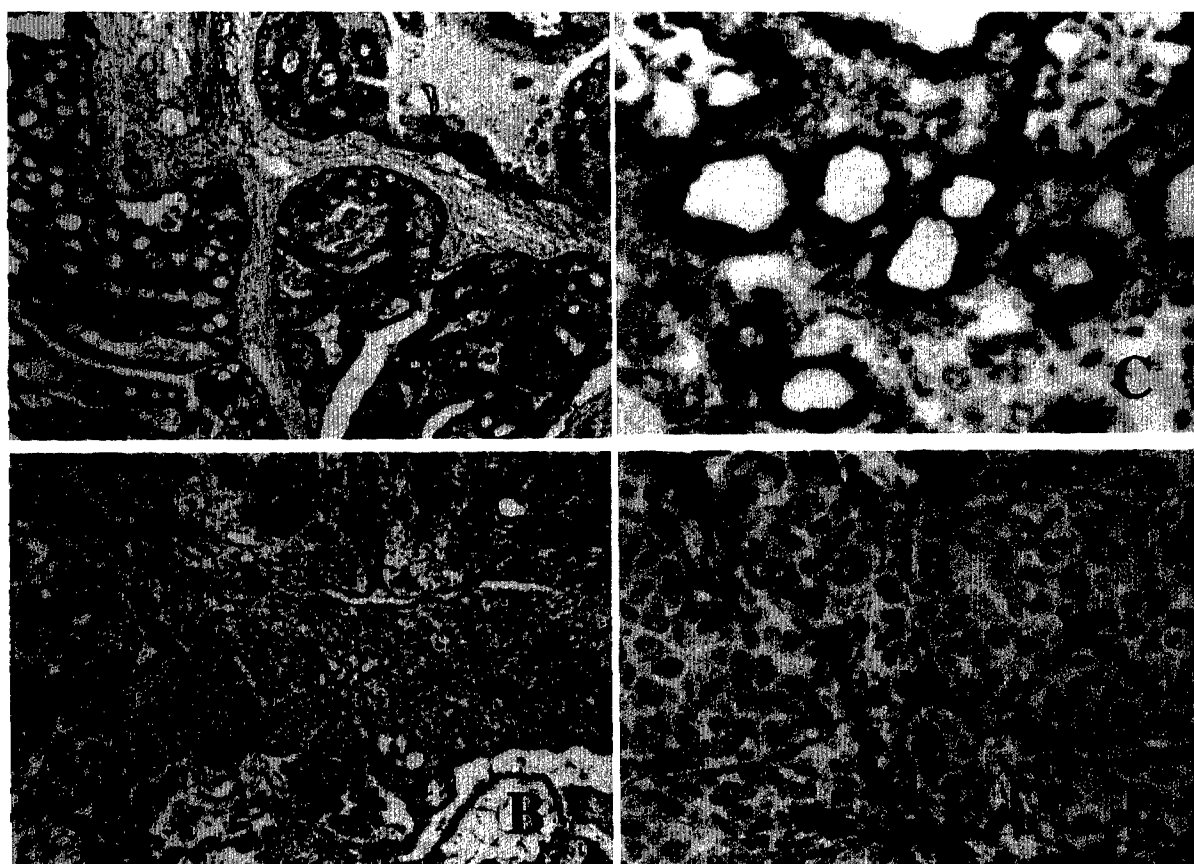

Figure 4 Immunohistochemistry on fixed *Wnt-1* TG p53+/+ and *Wnt-1* TG p53−/− mammary tumor sections with alpha smooth muscle actin antibodies. Panels (a) and (c) show a representative *Wnt-1* TG p53+/+ mammary tumor at 100× and 400×magnification, respectively. Panels (b) and (d) show a representative *Wnt-1* TG p53−/− mammary tumor at 100× and 400×magnification, respectively. The intense brown staining in the glandular structures of the p53+/+ tumors and comparatively low levels of staining in the more disorganized structures of the p53−/− tumors is consistent with the increased levels of alpha smooth muscle RNA and protein in the p53+/+ tumors as assayed by the Northern and Western blot analyses

genes and genes which regulate reactive oxygen species formation, have not been identified as differentially expressed genes in any of our tumor screens. This finding agrees with earlier findings by Yu *et al.* (1999) that the responses by p53 targets can vary considerably from cell line to cell line. Moreover, because in our model p53 is expressed at physiological levels in an *in vivo* heterogeneous context of mixed cell types, it is not surprising that the range of p53 targets observed is quite different from the cell based screens. However, the specific nature of the genes that are differentially expressed in our model is consistent with their playing a direct role in modulating the biological properties of the tumors. The potential relevance of each differentially expressed gene to the tumorigenesis process is discussed below.

Differentially expressed growth regulatory genes

p21$^{WAF1/CIP1}$ p21$^{WAF1/CIP1}$ is a protypical p53 target gene activated by p53 in response to a variety of cell stresses (Gorospe *et al.*, 1999). It is a cyclin-dependent kinase inhibitor which has both G1 and G2 checkpoint functions in response to DNA damage (Harper *et al.*, 1995; Dulic *et al.*, 1998; Bunz *et al.*, 1998). Its higher levels of expression in the presence of p53 may directly reduce tumor growth rates as observed in previous studies on the *Wnt-1* TG model. In these studies we found that *Wnt-1* TG p21 +/− mammary tumors had dramatically higher growth rates compared to *Wnt-1* TG p21 +/+ tumors (Jones *et al.*, 1999). In addition, maintenance of G1 and G2 checkpoints in the p53 +/+ tumors (in part through increased p21) may also contribute to the relatively high levels of genomic stability observed in this category of tumors.

Cyclin B1 Cyclin B1 is the major cyclin component of the mitotic cdc2-cyclin B complex initiating mitosis in eukaryotic cells (Musunuru and Hinds, 1997). It is upregulated in expression in the G2/M phase of the cell cycle. Recently, it has been demonstrated that p53 directly represses transcription of the cyclin B1 gene and thus may affect G2/M transition by reducing intracellular cyclin B1 levels (Innocente *et al.*, 1999; Taylor *et al.*, 1999). p21 has also been shown to inhibit the kinase activity of the cyclin B1-cdc2 complex (Xiong *et al.*, 1993; Harper *et al.*, 1995) and so p53 may mediate the G2 checkpoint through multiple mechanisms. Such mechanisms may contribute to the reduced rate of cell cycle progression and increased genomic stability observed in the p53 +/+ tumors (Donehower *et al.*, 1995; Jones *et al.*, 1997).

Cyclin G1 Cyclin G1 has been shown to be transcriptionally activated by p53 in response to DNA damage (Okamoto and Beach, 1994). The role of cyclin G1 in cell cycle control has not been established and published reports are contradictory as to whether cyclin G1 is growth promoting or growth inhibitory (Smith *et al.*, 1997; Shimizu *et al.*, 1998). Recently, however, it has been shown that overexpression of cyclin G augments the apoptosis process (Okamoto and Prives, 1999). However, since apoptosis levels are low in the p53 +/+ tumors, it is not clear how increased cyclin G1 levels in these tumors might affect their biological properties.

c-kit c-*kit* encodes a membrane tyrosine kinase receptor and is not known to be a direct target of p53. Its ligand is stem cell factor, which promotes growth in a number of hematopoietic precursor types (Ashman, 1999). Mutated versions of kit can be oncogenic and it is expressed at high levels in small cell lung carcinomas (Hibi *et al.*, 1991). However, in other types of human cancers, such as melanomas, thyroid carcinomas, and breast cancers, c-*kit* expression was reduced as the tumors progressed from normal tissues to benign lesions to malignant cancers (Natali *et al.*, 1992a,b, 1995). Moreover, ectopic expression of c-*kit* in breast cancer cells suppressed their growth (Nishida *et al.*, 1996), indicating that in mammary cells, c-*kit* may act as a tumor suppressor. Thus, in our mammary cancer model, the increased expression of c-*kit* in the p53 +/+ tumors may not only be a marker for a less malignant status, but may also be directly active in suppressing tumor cell growth rates.

Differentially expressed differentiation markers

Alpha smooth muscle actin Alpha smooth muscle actin is a major component of microfilaments and assists in maintaining cell shape and movement. It has been shown to be a p53 target gene (Comer *et al.*, 1998) and is also a marker for the myoepithelial cell compartment of the mammary gland (Gugliotta *et al.*, 1988). Alpha smooth muscle actin is downregulated in transformed cells and an inverse relationship between cellular proliferation and alpha smooth muscle actin expression has been widely observed (Leavitt *et al.*, 1985; Owens *et al.*, 1986). In the p53 +/+ tumors, it appears to be highly expressed in myoepithelial cells, a compartment which is present in lower quantities in the p53 −/− tumors (Figure 4). Thus, higher expression levels of this p53 responsive gene is a good indicator of retention of more differentiated cell types in the p53 +/+ tumors. Whether alpha smooth muscle actin has a role in the inhibition of tumor growth rates is unclear.

Cytokeratin 19 Cytokeratin 19 is one of the constituents of the intermediate filaments of epithelial cells and has recently been shown to be a p53 target gene (Zhao *et al.*, 2000). Cytokeratin 19 is a widely used luminal cell epithelial marker which is expressed at high levels in both normal and malignant human mammary epithelial cells (Moll and Schramm, 1998). It is expressed at high levels in many of the p53 +/+ tumors, indicating the presence of significant numbers of luminal epithelial cells in the tumors. However, the p53 −/− tumors display lower levels of cytokeratin 19 mRNA and protein, indicating that either these tumors have lost most of their luminal epithelial cells or that the luminal epithelial cells in these tumors have somehow lost cytokeratin 19 expression.

Kappa casein Kappa casein is not considered to be a p53 target, but is a milk protein specific for secretory alveolar cells in the mammary gland (Ginger and Grigor, 1999). It is found at high levels in normal breast tissue, lower levels in benign lesions, and not at all in invasive carcinomas (Rudland *et al.*, 1993). Its increased levels in the p53 +/+ tumors are consistent with the relatively differentiated state of these tumors. It also indicates retention of some functional secretory

alveolar cells in the p53+/+ tumors and their loss in the more dedifferentiated p53−/− tumors.

Conclusions

The identification of differentially expressed growth-related genes in our model is consistent with the observed differences in tumor growth rates between the p53+/+ and p53−/− mice. Interestingly, three of four of these genes are p53 target genes, indicating that wild type p53 is actively regulating expression of these genes. We hypothesize that such p53 signaling inhibits cell cycle progression in the p53+/+ tumor cells and may be at least partially responsible for their slower growth rate. The retention of G1 and G2 checkpoint control in the p53+/+ tumors may also contribute to the slower growth rate and delayed tumor incidence by preventing genomic instability and the resultant increase in oncogenic mutations.

The higher expression of differentiation markers in the p53+/+ tumors, such as alpha smooth muscle actin and kappa casein, is consistent with their more differentiated histopathological appearance. Since these two genes have been associated with myoepithelial and secretory alveolar cell types, respectively, it is likely that such differentiated cell types are lost in the progression of the p53−/− tumors to a more dedifferentiated state. In human breast neoplasms, benign lesions show retention of myoepithelial and secrectory alveolar cells, while in invasive carcinomas they are almost completely lost (Rudland et al., 1993). Thus, the p53+/+ tumors are likely to represent a more benign stage of mammary tumor progression, while the p53−/− tumors may be models for the more invasive stages of mammary carcinomas.

Another interpretation of the p53+/+ and p53−/− tumor differences is that they are derived from fundamentally different cells of origin. Wynford-Thomas has noted that about one-third of human invasive ductal breast cancers fall into a more aggressive subgroup which can be defined by poor differentiation, high proliferative rate, and estrogen receptor negativity (Wynford-Thomas, 1997). It was suggested that this subgroup may have arisen from a less-differentiated breast epithelial type such as a mammary stem cell. Interestingly, this aggressive subgroup of breast cancers has also been shown to correlate with a very high rate of p53 mutation (Thor et al., 1992; Mazars et al., 1992). The predominant, well-differentiated 'luminal' tumor type in humans and the Wnt-1 TG p53+/+ model may retain wild type p53 because there is little selective advantage for p53 mutation, whereas mutation of p53 in the mammary stem cells may provide a more profound advantage. This model is consistent with the idea that the Wnt-1 TG p53−/− tumors arise from an undifferentiated mammary stem cell while the Wnt-1 TG p53+/+ tumors arise from a more differentiated mammary cell type.

Despite the limitations inherent in doing expression analyses on end stage heterogeneous tumors, differentially expressed genes were identified in multiple p53+/+ and p53−/− tumors. The differentially expressed genes that were obtained were either growth regulators or indicators of cell differentiation status and were consistent with the differential histopathology

and biological properties of the p53+/+ and p53−/− tumors. Moreover, these results and the fact that many of these genes were *bona fide* direct transcriptional targets of p53 lends support to our argument that screening of whole tumors is a viable approach for identifying such genes. Further screens with large cDNA arrays should reveal additional differentially expressed genes. A remaining challenge will be to determine which of these differentially expressed genes have a direct effect on the biological properties of the mammary adenocarcinomas.

Materials and methods

Tumor samples

The Wnt-1 TG/p53 mammary cancer model from which the mammary tumors have been obtained has been previously described (Donehower et al., 1995). Wnt-1 TG p53+/+, Wnt-1 TG p53+/−, and Wnt-1 TG p53−/− females were monitored for tumors on a weekly basis from the time of weaning until the first observation of tumors. Four weeks after first observation of a tumor, the tumor bearing animal was sacrificed and the tumor excised. The skin and connective tissue were removed carefully and part of the tumor was placed in 10% neutral buffered formalin and the remainder was frozen in an Eppendorf tube at −80°C. The tumor segment in formalin was then fixed in paraffin and hematoxylin and eosin stained slides made from 4 μ sections of tumor tissue. These sections were then typed by histopathological examination and virtually all were categorized as Dunn type B mammary adenocarcinomas. In preparation for the various RNA assays described below, mRNA was purified from frozen tumor segments utilizing the Invitrogen mRNA extraction kit according to the manufacturer's specifications.

Differential display

The Clontech Delta Differential Display kit was used to screen tumor RNAs derived from p53+/+ and p53−/− mammary tumors. RNAs from p53+/− tumors that had lost their remaining wild type p53 allele (p53+/− LOH) were also utilized. These tumors were similar in their histopathological and biologic properties to p53−/− tumors. The protocols were all performed according to the manufacturer's instructions and will only be outlined here. Initially, the first strand cDNA is synthesized from each of the tumor RNA populations of interest, using murine leukemia virus reverse transcriptase and oligo(dT) as a primer. For differential display PCR, 10 arbitrary 5′ primers (oligo(dT)$_9$-NN, where N=A,G, or C) were combined with 10 arbitrary 3′ primers randomly in a PCR reaction in the presence of alpha-^{33}P-dATP. To resolve the PCR-amplified labeled cDNA fragments, a denaturing 5% polyacrylamide/8 M urea gel was used. After electrophoresis, the denaturing gels were subjected to autoradiography and the X-ray films were carefully examined for differentially expressed bands. Generally, multiple tumor RNAs of each genotype were run in parallel to identify fragments which were consistently over-expressed or underexpressed in a particular genotype. Once differentially expressed bands were identified, they were excised from the gel, placed in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and the cDNA fragments were eluted from the gel slice by boiling. The DNA was then reamplified using the original 5′ and 3′ arbitrary primers. ^{32}P-labeled probes were made from the reamplified fragments by the random primed oligo labeling procedure using the Roche High Prime kit and used to probe Northern blots of RNAs from 6–8 p53+/+ and 6–8 p53−/− tumors. Those probes

that showed consistent p53 genotype-specific overexpression or underexpression were ligated into a Clontech TA cloning vector using the Clontech AdvanTAge PCR cloning kit according to the manufacturer's specifications. Positive clones were amplified and sequenced with the M13 forward primer and the Amersham Sequenase kit. About 200–300 base pairs of insert sequences were identified by this method and these were used to probe GenBank in homology searches.

Northern blot hybridization

For Northern blot analysis, the Ambion NorthernMax kit was used according to manufacturer directions. Two μg of mRNA from 5–8 p53+/+ tumors and 5–8 p53−/− tumors were loaded in each well of the agarose gel. After electrophoresis, the separated RNAs were transferred to a Zeta-Probe membrane from Bio-Rad. Labeled ^{32}P probes for each differentially expressed gene were hybridized to the membranes. After hybridization, membrane washing, and autoradiography, the band hybridization intensities on the filters were quantitated on the Molecular Dynamics Storm 860 Phosphorimager. The filters were then stripped and reprobed with a labeled GAPDH probe to provide a normalization control. After autoradiography, this filter was also subjected to phosphorimager analysis. Relative hybridization intensities for a particular gene in a tumor were always normalized to the intensity of the GAPDH signal in that tumor to obtain quantitative values.

cDNA arrays

The Clontech Atlas mouse array I with 588 known cDNAs attached to duplicate nylon membranes was used for the cDNA array screen. One μg of mRNA from a p53−/− tumor and a p53+/+ tumor were each reverse transcribed in the presence of alpha ^{32}P-dATP. The labeled cDNA populations were then each hybridized overnight to the two array filters according to the manufacturer's specifications. After washing and autoradiography, the hybridization intensity of each of the genes on the filter was quantitated by phosphorimager analysis. Spot intensities were then normalized to the intensities of housekeeping genes on the filter to estimate relative hybridization levels between the p53+/+ and p53−/− filters. Genes that repeatedly showed more than 2.5-fold differences in hybridization intensity between the two filters were assessed for differential expression by Northern blot hybridization after synthesis of probes by RT–PCR using gene specific primers.

RNAse protection assay

RNA expression levels of 14 cyclin genes in the p53+/+ and p53−/− tumors were assessed using two Pharmingen multiprobe RNAse protection assay kits (mcyc-1 and mcyc-2). ^{32}P-labeled RNA mouse cyclin probes were generated by T7 RNA polymerase-directed synthesis of 14 different cyclin gene templates. The multi-probe set was then hybridized in excess to target RNA (10 μg mRNA for each sample) in solution, after which free probes and other single-stranded RNA are digested with RNAses. The remaining RNAse protected probes were purified, resolved on denaturing polyacrylamide gels, and quantified by phosphorimaging. The quantity of each mRNA species in the original RNA sample could then be determined based on the intensity of the appropriately sized protected probe fragment following normalization to a control probe (GAPDH) used along with the cyclin probes.

Immunoblot assays

Western blot analysis of alpha smooth muscle actin, cytokeratin 19, c-kit, cyclin B1 and cyclin G1 protein was performed from tumor lysates of multiple p53+/+ and p53−/− tumors. For alpha smooth muscle actin protein detection, 20 μg of total tumor lysate was run on an 8% SDS polyacrylamide gel. The gel was transferred to a 0.45 μm pore size nitrocellulose membrane (BA85, Schleicher & Schuell) for 2 h at 75 volts and then blocked with 5% nonfat dry milk in Tris-buffered saline with 2% Tween 20 (TBST) overnight at 4°C. The blot was incubated with mouse monoclonal antibody for alpha smooth muscle actin (Clone 1A4 from NeoMarkers) diluted at 1:1000 in TBST with 1% nonfat dry milk for 1 h at room temperature. After washing three times with TBST, the blot was incubated with goat anti-mouse IgG2a peroxidase. Protein was detected using the supersignal enhanced chemiluminescence (ECL) system (Pierce). For cytokeratin 19 detection, 50 μg protein lysate was loaded for gel separation. The antibody used for cytokeratin 19 was Clone A53-B from NeoMarkers. Dilution and incubation conditions were the same as for alpha-smooth muscle actin. c-kit antibody (M-14, Santa Cruz) was diluted 1:200. Antigoat IgG was diluted 1:2000 as secondary antibody. For cyclin B1 detection, 20 μg protein lysate was loaded on an SDS-polyacrylamide gel. After transfer to a nitrocellulose membrane, the blot was incubated with a polyclonal antibody to cyclin B1 (Oncogene Research Ab-3) diluted 1:2000. For cyclin G1 analysis, 100 μg of tumor lysate was loaded on a 12% SDS-polyacrylamide gel. Cyclin G1 antibody (C-18, Santa Cruz) was diluted 1:200 for the Western blot. Goat anti-rabbit antibody (SC-2030, Santa Cruz) was diluted 1:1000 as secondary antibody. Each blot was stripped and reprobed with an antibody against all six isoforms of vertebrate actin (C4, Boehringer-Mannheim) as a loading control.

Immunohistochemistry on tumor sections

Tumor samples were fixed with neutral buffered formalin and embedded in paraffin. Tumor slides were deparaffinized, rehydrated, and treated with 3% H_2O_2 to quench any endogenous peroxidase. Samples were then boiled in 10 mM sodium citrate buffer (pH 6) for 10 min to unmask the antigen. After washing with PBS, the slides were blocked with 10% goat serum at room temperature for 30 min and then incubated with 1:200 mouse monoclonal antibody (1A4, Neomarkers) at 4°C overnight. After warming up to room temperature, the slides were washed with PBS and incubated with 1:400 peroxidase-coupled secondary antibody (goat anti-mouse, Boehringer-Mannheim) for 1 h. The antibody-antigen reaction was visualized by DAB staining and counterstained with hematoxylin.

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